DTU Aqua Internship: effects of fatty acids on fish behaviour, and how size affects Rainbow Trout swimming speed

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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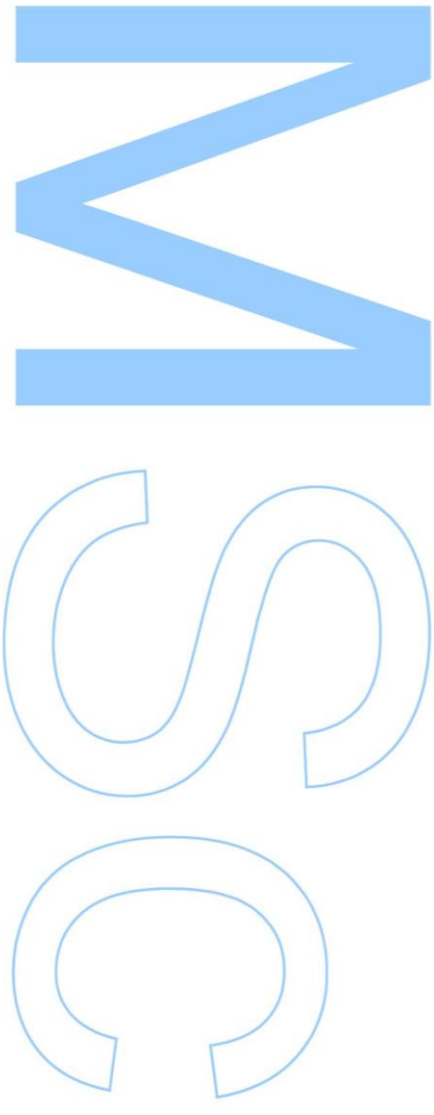

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"The ending is always a surprise."

Daniel Wallace, Big Fish

Summary

This internship was done in partnership with DTU Aqua and took place at their facilities in the North Sea Science Park in Hirtshals, Denmark. This report focuses mainly on my contributions in various projects and experiences. The internship started with daily routine, followed by running two experiments on Pikeperch fry (*Sander lucioperca*), on fast-response and rheotaxis, for an ongoing project on Pikeperch nutrition. The aim was to investigate the effects of increasing inclusion of phospholipids and additional effect of n3-/n6 HUFA ratios on Pikeperch larval and/or juvenile physiology and welfare indicators. During this task, the neural development was measured using visual and mechano-sensory acuity during avoidance responses and rheotactic behaviour.

Afterwards, I was assigned the task of analysing videos from hypoxia experiments previously conducted on Meagre (*Argyrosomus regius*). The aim of this was to determine whether fatty acid nutritional history had an effect on the stress response and subsequent recovery in individual fish. The trial presented no statistical differences across the 3 diets in any of the variables analysed. The lack of differences suggests fatty acids have little or no impact on the responsiveness of fish to stress, but it could also mean that (1) the duration, intensity and recovery of the stress response does not show the true effects of lipid inclusions on stress, or (2) the type of lipids used are not the most impactful on the stress response.

Lastly, I worked on determining the effects of fish size on optimal swimming speed and cost of transport in rainbow trout (*Oncorhynchus mykiss*), and implications for their use as variables in exercise induced growth improvement. All differences in variance between groups were evaluated using *F*-tests, and single linear regression analysis by using the "least squares" method was used to compare optimal swimming speed (Uopt) and critical swimming speed (Ucrit), in body lengths per second (BL/s), with body mass (BM), standard length (SL), Blocking index, stride length, tailbeat frequency (TBF), hepatosomatic index (HSI), viscerosomatic index (VSI), tailbeat amplitude (TBA) and red muscle index (RM). Both Uopt and Ucrit showed similar interactions with the variables in question. These were found to be negatively correlated with BM, SL, and Blocking index, and positively correlated with stride length and TBF ($p < 0.05$). No correlation was found between speed and HSI, VSI, TBA and RM ($p > 0.05$). We concluded that smaller trout can achieve higher critical swimming speeds and have a proportionally higher optimal swimming speed. The main reason for this appears to be the ability to reach higher tail beat frequencies in combination with a greater stride length, which in turn results from less hydrodynamic drag.

Resumo

Este estágio foi realizado em parceria com o DTU Aqua e teve lugar nas suas instalações no North Sea Science Park em Hirtshals, Dinamarca. Este relatório foca-se principalmente nas minhas contribuições em vários projetos e experiências. O estágio começou com trabalho de rotina, seguido de duas experiências em lúcio perca (*Sander lucioperca*), em resposta-rápida e reotaxia, para um projeto a decorrer sobre nutrição desta espécie. O objetivo era investigar os efeitos de maiores inclusões de fosfolípidos e o efeito adicional de índices de n3-/n6 HUFA em morfologia larvar/juvenil e indicadores de bem-estar de lúcio perca. Durante este projeto, o desenvolvimento neural foi medido usando acuidade visual e mecano-sensorial durante respostas de fuga e comportamento em reotaxia.

Posteriormente, tratei da análise de vídeos de experiências em hipoxia previamente realizadas em Corvina (*Argyrosomus regius*). O objetivo era determinar se o historial nutricional de ácidos gordos tinha algum efeito na resposta ao stress e subsequente recuperação em peixes individuais. O ensaio não apresentou diferenças estatísticas entre as 3 dietas em qualquer uma das variáveis analisadas. A falta de diferenças sugere que os ácidos gordos têm pouco ou nenhum impacto na capacidade de resposta a stress e recuperação, mas pode apenas significar que (1) a duração e intensidade da resposta ao stress e recuperação não demonstram os verdadeiros efeitos das inclusões lipídicas no stress, ou (2) o tipo de lípidos usados não são os que mais impacto têm na resposta ao stress.

Por fim, trabalhei em determinar os efeitos do tamanho de Truta Arco-íris (*Oncorhynchus mykiss*) em velocidades de natação ótimas e em custo de transporte, e implicações para o seu uso como variáveis na melhoria do crescimento induzido pelo exercício. Todas as diferenças de variância entre grupos foram avaliadas através de teste-F, e foi feita uma análise de regressão linear usando o método "mínimos quadrados" para comparar velocidade ótima de natação (Uopt) e velocidade crítica de natação (Ucrit), em comprimentos corporais por segundo (BL/s), com massa corporal (BM), comprimento padrão (SL), índice de bloqueio, comprimento do passo, frequência da batida da cauda (TBF), índice hepatossomático (HSI), índice viscerossomático (VSI), amplitude da batida da cauda (TBA) e índice de músculo vermelho (RM). Uopt e Ucrit mostraram interações similares com todas as variáveis em questão. As velocidades estão negativamente correlacionadas com BM, SL e índice de bloqueio, e correlacionados positivamente com o comprimento do passo e TBF ($p < 0.05$). Não se viu correlação entre as velocidades e HSI, VSI, TBA e RM ($p > 0.05$). Concluímos que uma truta menor pode atingir

velocidades críticas de natação mais altas e ter uma velocidade de natação ideal proporcionalmente maior. O principal motivo para isso parece ser a capacidade de alcançar uma TBF mais elevada em combinação com um comprimento de passo maior, o que, por sua vez, resulta de um menor arrasto hidrodinâmico.

Keywords

Aquaculture; RAS; Meagre; Stress; Behavioural response; Fatty acids; Pikeperch; Rheotaxis; Fast-escape; Phospholipids; HUFA; Rainbow trout; swimming speed; Size.

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Abbreviations

RAS- Recirculating Aquaculture Systems

DTU- Danish Technical University

HUFA- Highly unsaturated fatty acids

DHA- Docosahexaenoic acid

EPA- Eicosapentaenoic acid

ARA- Arachidonic acid

PL- Phospholipids

Dph- Days post hatching

BL- Body lengths

Uopt- Optimal swimming speed

Ucrit- Critical swimming speed

BM- Body mass

SL- Standard length

HSI- Hepatosomatic Index

VSI- Viscerosomatic Index

TBF- Tailbeat frequency

TBA- Tailbeat amplitude

RM- Red Muscle

SGR- Standard growth rate

Introduction

Aquaculture is already one of the world's fastest growing animal production industries, and still has a lot of potential for growth. Global capture production has reached its limit of around 90 million tonnes, but aquaculture production has maintained a constant annual growth reaching a global production of about 70 million tonnes in 2013 (FAO, 2014). However, aquaculture requires continuous development of new technologies to further increase cost effectiveness, better answer consumer demands, focus on sustainability and improve food safety. This constant growth came at a cost, though, and raised a number of issues that became the focus of today's aquaculture research.

Aquaculture's environmental impacts

Aquaculture practices and their environmental impacts on ecosystems vary widely, and usually attract the consumers' attention. Greater impacts can be found where aquaculture is concentrated and where nutrient exports exceed carrying capacity. While individual intensive/large-scale farms require environmental impact assessments (EIAs), licensing and certification systems, there are no management measures covering the overall impact of small farms. Some farms generate impacts that affect the farming systems themselves by causing hypoxia, fish kills, fish stress, facilitating conditions for spreading diseases, etc. (White et al., 2008).

These impacts result mostly from untreated wastewater laden with uneaten feed and fish feces, which contributes to nutrient pollution near coastal ponds and cages (Buschmann et al., 2006; Taranger et al., 2015). Pollution problems are most severe in shallow or confined water bodies (Iwama, 1991); they also tend to be serious in regions where intensive aquaculture systems are concentrated. In many such areas, sedimentation of food particles and fecal pellets under and around fish pens and cages negatively affects the biogeochemistry of benthic communities (Ervik et al., 1997). Moreover, nitrogen wastes (for example, ammonia and nitrite) that exceed the assimilative capacity of receiving waters lead to deterioration in water quality, that is toxic to fish and shrimp (Hargreaves, 1998). For instance, the fast-growing Vietnam catfish has attracted strong criticism based on alleged environmental and food safety issues. Although many of the accusations may not be supported (Little et al., 2012), the local eutrophication impacts cannot be denied, and the role of aquaculture in eutrophication has been demonstrated. One study by Bouwman et al. (2013) found that freshwater aquaculture adds to the nutrient loading of river systems, which is likely to increase in the future.

Another way aquaculture negatively affects the environment is through biological pollution. Atlantic salmon—the dominant salmon species farmed—frequently escapes from net pens. In 1993, Hansen et al. (1993) estimated that as much as 40% of Atlantic salmon caught by fishermen in areas of the North Atlantic Ocean were of farmed origin. In the North Pacific Ocean, by 1997, over 255,000 Atlantic salmon had reportedly escaped since the early 1980s and were caught by fishing vessels from Washington to Alaska (McKinnell & Thomson, 1997). Increasing evidence suggests that farm escapees may hybridize with and alter the genetic makeup of wild populations of Atlantic salmon which are genetically adapted to their natal spawning grounds (Gross, 1998). Such genetic alterations could exacerbate the decline in many locally endangered populations of wild Atlantic salmon (Gross, 1998; McGinnity et al., 1997; Slaney et al., 1996).

Lastly, the continuous use of fish and fish byproducts in aquaculture feeds has had its fair share of impacts. In the early 1990's, two papers (New & Wijkstrom, 1990; Wijkstrom & New, 1989) expressed concern about the use of marine resources for feeds and coined the term "fishmeal trap" which became a common parlance in aquaculture. They stated that, at some point in the future, farmers cultivating shrimp and carnivorous fish would run into a cost-price squeeze - the fishmeal trap - and that it might be the first of several ingredient traps which might constrain certain forms of aquaculture in the future. Their prediction was correct and nowadays aquaculture research is greatly focused on solving this issue.

Fishmeal trap

If aquaculture continues to expand as it has been, its growth will either be restricted by the limited supplies of fishmeal and fish oil or the high demand for these resources will result in increased pressure on wild feed fish stocks. There are approximately 300 dedicated plants worldwide that produce about 6.3 million tons of fishmeal and 1.1 million tons of oil annually from roughly 33 million tons of whole fish and trimmings (Shepherd & Jackson, 2013). At first glance, small pelagic species appear to represent the bulk of these "industrial/reduction captures" (between 20% and 30% of the total fish landings) (Alder & Pauly, 2006) and aquaculture production is often presented as a solution to this economic, social and biological problem, as it can be considered a close substitute for marine capture fisheries. However, these small fish are still an important part of marine ecosystems, and fishing these for the sole purpose of feed production can have a significant impact on worldwide fisheries.

In 2007, the proportion of the cultivated fish indigenously carnivorous was 31 percent while 69 percent were non-carnivorous species (Nordahl, 2011). The digestive system

of carnivorous fish is not accustomed to untreated plant materials and therefore the first aquaculture farmers used feed consisting mainly of fishmeal, which is ground and dried fish, in addition to fish oil. Non-carnivorous species were also fed fishmeal because it enabled more rapid and stable biomass growth than diets consisting only of vegetable meals (Naylor et al., 2000). Global demand for fishmeal and fish oil has been increasing, as have their prices (Figures 1 and 2), making these no longer low-value products.

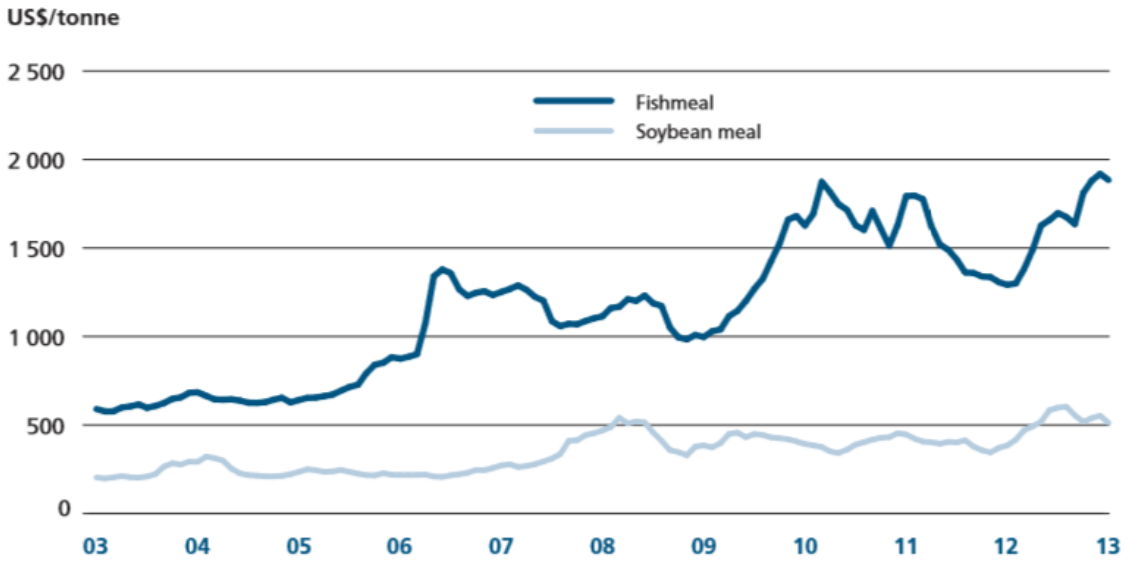

Figure 1: Trends in the price of fishmeal and soybean meal between 2003 and 2013. Source: FAO. 2013. FAO Fisheries and Aquaculture Information and Statistics Branch. Rome.

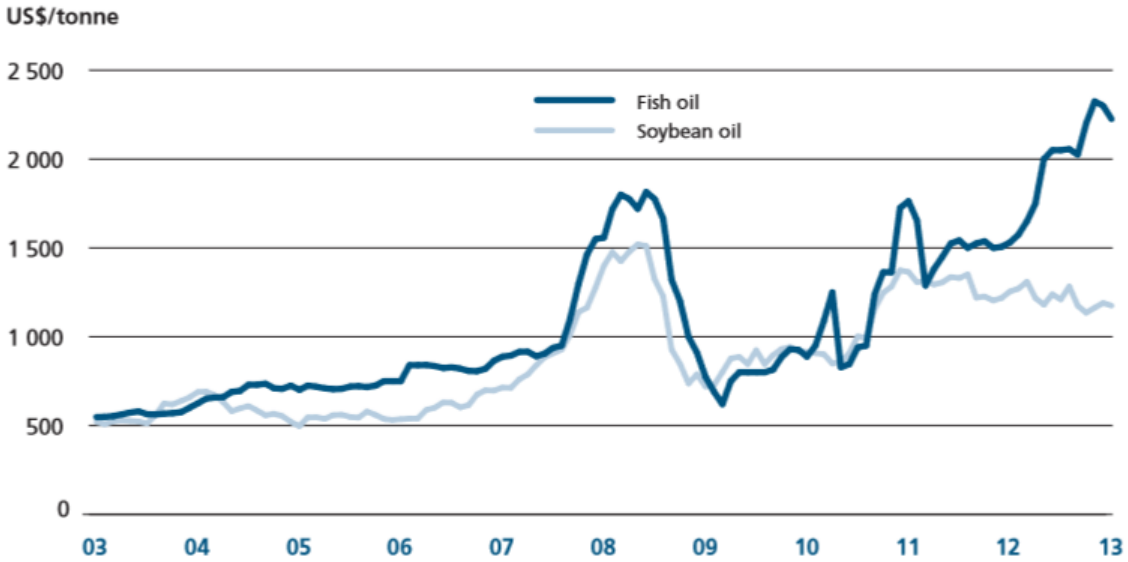

Figure 2: Trends in price of fish oil and soybean oil between 2003 and 2013. Source: FAO. 2013. FAO Fisheries and Aquaculture Information and Statistics Branch. Rome.

However, the use of these fish byproducts has been decreasing slightly in recent years, according to IFFO statistics, mostly due to more efficient use and the increasing substitution of fish by vegetable protein and oil. In the graph from IFFO (2013), the green line shows the growth of fed aquaculture since 2000, while the red and yellow dotted

lines show the use of fishmeal and fish oil during the same period. While the use of fishmeal has increased since the year 2000, it has remained somewhat stable since 2004. However, fish oil use has remained constant since the year 2000, only slightly decreasing in 2010.

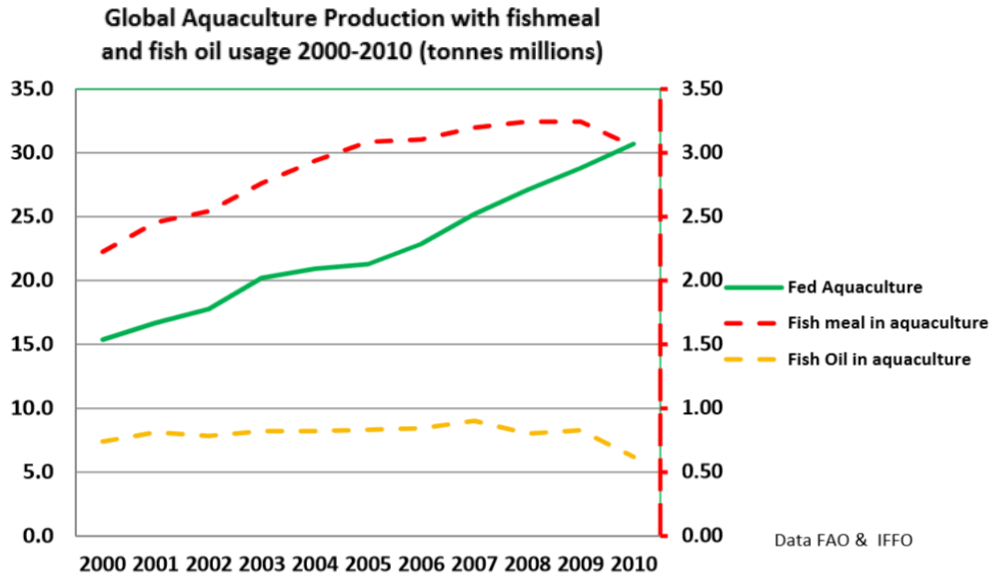

Figure 3: Fishmeal and fish oil consumption in relation to growth of 'fed' aquaculture (IFFO, 2013).

This supports the idea that the expansion of aquaculture is not resulting in an increasing use of fishmeal and fish oil. While these ingredients' great qualities are still valued, producers of feeds were familiar with their limited availability, and addressed this problem accordingly.

Recirculating Aquaculture Systems

Compared to traditional systems recirculating aquaculture systems (RAS) lessens part of the environmental effects above described, such as excessive use of fish meal and oil as feed ingredients (Naylor et al., 2000), escapees of farmed fish from sea cages into the wild and the discharge of waste into the environment (Buschmann et al., 2006; Taranger et al., 2015). Moreover, cage-based and flow-through aquaculture systems face obstacles for their expansion, such as space limitations due to competition with other activities and industries, and limited clean water availability. Therefore, some European countries are taking a step forward to further implement this technology in large scale aquaculture production.

Recirculating aquaculture systems (RAS) are technological systems for farming fish or other aquatic organisms by completely or partially reuse the production water by

circulating it through a treatment system that reduces biological and chemical waste loads to levels comparable to the influent water (Fisher, 2000), enabling 90-99% of the water to be recycled. Recirculation systems use a series of processing stages that provide some or all of the following: separation of large suspended solids (uneaten food and faeces); fine filtration; processing to reduce suspended solids, chemical oxygen demand and dissolved organic carbon, and to re-oxygenate the water; removal of dissolved organic materials (foam fractionation); disinfection; biological filtration/ nitrification/ denitrification; and toxicant removal with activated charcoal (Fisher, 2000). The development of recirculating aquaculture systems (RAS) is particularly relevant for urban areas, because RAS is highly productive and can be located virtually anywhere, relatively independent of climate and water resource availability (Medina et al., 2016). RAS production has grown a lot since their introduction in the late 1980's, not only in volume, but also in species diversity, and today more than 10 species are produced in these systems (Martins et al., 2005; Rosenthal, 1980; Verreth & Eding, 1993; Martins, 2010).

In Denmark, government pressure and rules limiting feeding stimulated the shift towards outdoor RAS (Pedersen et al., 2008), resulting in an aquaculture industry "characterized by recycling systems" (Badiola et al., 2012). Here, aquaculture is predominated by the rainbow trout (*Onchorhynchus mykiss*) culture and more than 10% is produced in RAS (Roque d'orbcastel et al., 2009), making this technology increasingly important in Denmark (Jokumsen & Svendsen, 2010). According to Statistics Denmark, the use of sea farms has been decreasing significantly, while RAS and traditional trout farms are slowly raising, making Denmark one of the most promising countries in sustainable aquaculture.

RAS have a number of advantages associated to them, such as a much lower use of water (Verdegem et al., 2006), easier ways to manage waste and nutrient recycling (Piedrahita, 2003), better hygiene and disease control (Summerfelt et al., 2009; Tal et al., 2009) and a lower chance of biological pollution (Zohar et al., 2005). Additionally, these systems can be built closer to markets (Masser et al., 1999; Schneider et al., 2010), reducing CO_2 emissions associated with transport (Martins et al., 2010). RAS can theoretically be used for any species grown in aquaculture and, depending on how much water is recirculated, can be carried out at different intensities. Water is an important issue nowadays, as it has become a limited resource in many regions, making recirculation advantageous to the environment. A vital sector of the aquaculture industry in which RAS technology is widely applied is hatchery production. Future water shortage, large season variation in water temperature and low inlet water quality are the main

factors driving the shift of smolts production from flow through to RAS in Norway (Kristensen et al., 2009), one of the most advanced countries in aquaculture production. In Norway, even though RAS are almost universally employed to produce salmon smolts, recent economic studies on land-based salmon systems show that salmon can actually be produced more cheaply in RAS, but the investment costs are still too high to justify major changes in the way salmon is currently grown in net-pen systems (Ebeling & Timmons, 2012).

The particularities of these systems have also shaped the feed industry, not only for the sake of the fish, but also for better maintenance. Only dry feed can be recommended for use in a recirculation system. The use of trash fish in any form must be avoided as it will pollute the system heavily and infection with diseases is very likely. The use of dry feed is safe and also has the advantage of being designed to meet the exact biological needs of the fish. In a recirculation system, a high utilization rate of the feed is beneficial as this will minimise the amount of excretion products thus lowering the impact on the water treatment system. The feed conversion rate (FCR) is improved, and the farmer gets a higher production yield and a lower impact on the filter system (Losordo et al., 1998; Roque d'orbcastel et al., 2009).It should be noted that feeds especially suitable for use in recirculation systems are available, which aim at maximising the uptake of protein in the fish thus minimising the excretion of ammonia into the water. Therefore, fine-tuning the feed is of major importance when using RAS, since it affects the system and the fish in various ways.

Recirculating systems are biologically intense, and fish must be reared intensively for recirculating systems to be cost effective (Losordo et al., 1992). It is also a very artificial environment, which makes it harder for fish to get accustomed. These issues, along with requiring a lot of maintenance and handling by trained professionals, cause stress and lower SGR in the cultured fish, along with other problems. Like other issues of RAS, addressing these complications is a focus of today's aquaculture research.

Nutrition and stress in aquaculture

As aquaculture grows, it faces new challenges every day and solving them is a priority to improve the industry. The main goal is to sustainably produce a healthy, tasteful and accessible food source, and, in order to do that, aspects like nutrition, water quality, fish density and others, need to be studied in depth to understand how each affect the fish itself and find solutions to the problems that arise.

The first aspect that comes to mind is fish nutrition. Nutrition influences growth, health, reproduction and response to physiologic and environmental stressors. Nutritional

requirements for most farmed fish species are not yet established, despite the heavy focus of research on this matter for the past decade. An important part of these nutritional studies is addressing how a diet might affect stress response.

Stress management is a big part of maintaining a healthy aquaculture. While severe stress may cause high mortalities in short term, sub lethal stress can impact physiological and behavioural functions at a longer term, leading to suboptimal production. Practices such has handling, sorting, grading, transport and poor water quality impose stress on fish (Barton & Iwama, 1991; Iwama et al., 2011; Schreck, 1982), so it's important to understand how to keep stress at its lowest. However, stress can be considered at different levels, either as individual fish or as a community, which makes it a difficult parameter to define and measure. However, in finfish aquaculture, studying the effects of stress is more upfront because they usually involve physiological responses to one or more stressors. Fatty acid enrichment has been shown to be useful for increasing stress resistance in larval marine fishes (Ako et al., 1994; Kraul et al., 1993). With the formulation of new diets, that use other sources of lipids (for example, vegetable oil) to reduce the use of fish oil, it is necessary to study how they could possibly affect these stress responses.

In order to improve overall aquaculture production, these and other aspects must be studied and understood, for aquaculture's well-known species and new species alike.

Swimming studies

Nowadays, research mostly focuses on studying the potential of species new to aquaculture or further developing heavily aquacultured species. Studying a well-known species is all about fine-tuning the different aspects of production, in order to obtain a cost-effective product. In the intensive conditions of RAS, fish are usually reared at high densities. It is therefore essential to further develop and understand ways to reduce the impacts of high density.

The use of a water current has shown an array of beneficial effects on several parameters of productivity and welfare of farmed salmonids (Davison & Herbert, 2013; McKenzie et al., 2012). These benefits can be physiological improvements as well as behavioural changes. One example is the display of schooling behaviour, that results in a reduction of aggressive behaviour, through a decrease of hierarchical interactions and lower stress levels (Adams et al., 1995; Brännäs, 2009; Christiansen et al., 1989). Even though swimming may be more energetically costly than non-swimming, research has found, through improved growth performance in a number of species, that compensatory gains make up for the added costs of swimming (Davison, 1997; East & Magnan, 1987;

Houlihan & Laurent, 1987; Jobling et al., 1993; Jørgensen & Jobling, 1993). Furthermore, swimming is likely to improve fillet taste and quality, because fish growth that occurs along with exercise may involve lower lipid deposition in the body since aerobic exercise is profoundly fuelled by lipids (Kieffer et al., 1998; Lauff & Wood, 1996; Lauff & Wood, 1997; Richards et al., 2002). However, this must be carefully interpreted because the influence may be hidden by several other factors, such as feeds, stress, filleting procedures, storage times and conditions and smoking procedures (Johnston et al., 2007; Rasmussen et al., 2011).

Species

Pikeperch *Sander lucioperca* (Linnaeus) is a highly valued species with great potential in European freshwater aquaculture (Craig, 2008; Philipsen & van der Kraak, 2008; Wang et al., 2009). It's a valuable species for aquaculture due to its rapid growth, flesh quality and high commercial value (Hamza et al., 2008b), and there's a growing interest in the development of pikeperch intensive rearing in several countries (Hamza et al., 2007; Kestemont et al., 2007; Breteler, 1989; Molnar et al., 2006; Ostaszewska et al., 2005; Ruuhijärvi et al., 1991; Zakes, 1999).

Former studies (Kamstra et al., 2001; Schulz et al., 2004; Zakes, 1997) have shown that pikeperch can be reared in recirculation system and fed by artificial diets very efficiently, but knowledge on the nutritional requirements of pikeperch is weak. However, more recent studies using commercial diets have indicated significant improvements in survival and growth with appropriate larval diets and/or optimal weaning timing (Kestemont et al., 2007; Ostaszewska et al., 2005). This shows how pikeperch production is still defining its bearings, and only now is it starting to move on to fine-tuning production practices.

Meagre (*Argyrosomus regius*) belongs to the *Sciaenidae* family and it is a carnivorous species that inhabits the Mediterranean and the Black Seas, and also occurs along the Atlantic coast of Europe (Whitehead et al., 1986). The fact it can adapt very easily to captivity, exhibiting a high growth rate and good feed conversion ratio (Jiménez et al., 2005), and has the ability to tolerate wide ranges of temperature and salinity, makes it a suitable candidate species for the diversification of aquaculture in the Mediterranean region (Quéméner et al., 2002). Moreover, its lean and high lipid quality flesh makes meagre a species with high nutritional value (Grigorakis et al., 2011; Monfort, 2010; Piccolo et al., 2008; Poli et al., 2003; Quéméner et al., 2002).

Aquaculture of meagre started in 1997 and European production in 2009 was 1912 t (Duncan et al., 2013). Production techniques are similar to those used for European

seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*). For the time being, a few attempts of meagre culture have been made by hatcheries in France and Greece, initially succeeding in its artificial propagation (Duncan et al., 2008; Poli et al., 2003). However, there is little information available on the dietary requirements of meagre, particularly on dietary lipid requirements.

Future studies are needed on the nutritional requirements and possible implications of nutrition on cannibalism and variable FCRs. Hatchery and juvenile production techniques need to be improved to optimise culture productivity and larval quality.

Rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) is the most widely cultured cold freshwater fish, and the second most important salmonid in global aquaculture, after Atlantic salmon, *Salmo salar* (Linnaeus) (Adeli & Baghaei, 2013). The EU, Iran and Turkey are the main producers, with the EU and Iran producing almost exclusively for domestic consumption (Adeli & Baghaei, 2013; EUMOFA, 2014; Kalbassi et al., 2013), but, according to FAO, more than 75 countries are officially represented in the rainbow trout production statistics. Total world production reached 500,000 MT for the first time in 2001 and was recorded in 2014 as 812,940 tons with an economic value of 3.3 billion euros (FAO, 2016).

The wide distribution of rainbow trout aquaculture is due to a number of biological advantages that make it a species easy to culture. The production of rainbow trout is practiced in different production systems including extensive flow through system, intensive flow through system and intensive recirculating aquaculture systems, and can therefore adapt to the placement and investment of the aquaculture. Furthermore, rainbow trout has a number of biological characteristics that make it truly appealing for aquaculture, such as rapid and high growth potential, ease of captive breeding, robustness of fry at first feeding, ability to withstand high density culture conditions without compromising growth and welfare standards, nutritionally rich flesh and tolerance to a wide range of environmental variables (Singh et al., 2017). The fact that it's an adaptable species made it a focus of aquaculture production and research all around the globe for many years now. This means that there is already a lot of information on this species, and research is so advanced it now focus on fine-tuning smaller aspects to further improve productivity.

Internship description

This internship was done in partnership with DTU Aqua and took place at their facilities in the North Sea Science Park in Hirtshals, Denmark. During 7 months, I had the chance to perform a number of experiments on my own, be a part of the daily routine, contribute in various projects, and work with professionals with a widespread knowledge on aquaculture. I worked closely with Peter Skov, Associate Professor at DTU Aqua, on different aspects of fish physiology that present current issues of modern day aquaculture.

Working Facilities

DTU Aqua has facilities specifically designed and well-suited for research within aquaculture at the North Sea Science Park in Hirtshals, Jutland. These provide the base for the institute's research into aquaculture, farming technology and fish physiology. The facilities enable DTU Aqua to work with a wide variety of species under very different conditions and are outstanding in a national and international context. They cover everything from small-scale pilot experiments to trials at commercial and semi-commercial scale.

The aquaculture facilities include a hatchery for salmonids and marine fish, larval rearing and copepod and rotifer breeding, facilities for studying nutrient balances and feed digestibility, a wide variety of recirculation systems, a respiration laboratory, a salt water model fish farm, laboratories for chemical analyses, water chemistry, hormones and amino acids, and a workshop.

The various recirculating systems include:

- Recirculation system for technology and method development
- Traditional recirculation systems for e.g. growth experiments
- Recirculation systems for measuring potential environmental impact etc.
- Respirometry system for physiological experiments, including measurement of energy balances, oxygen consumption under different rearing conditions, and assessment of the nutritional value of the feed
- System for studying behaviour, welfare etc.

Daily Routine and Tasks

During this internship I got the chance to work with lesser-known species, such a pikeperch and meagre, and, on the other hand, a well-known species, rainbow trout.

The daily routine consisted of feeding pikeperch fry (*Sander lucioperca*) and European lobster fry (*Homarus gammarus*), cleaning their tanks/trays, and helping the technicians with other maintenance tasks. These included a number of tasks, such as managing feeds for digestibility trials, relocating fish, helping in tank weighings, etc.

Afterwards, I started running two experiments on Pikeperch fry, on fast-response and rheotaxis, for an ongoing project on Pikeperch nutrition. DTU Aqua aimed to investigate the effects of increasing inclusion of dietary phospholipids and additional effect of n3-/n6 HUFA ratios on Pikeperch larval and/or juvenile physiology and welfare indicators, such as ontogenetic development and performance. During this task, the neural development was measured using visual and mechano-sensory acuity during avoidance responses and rheotactic behaviour.

After that, I was assigned the task of analysing videos from hypoxia experiments previously conducted on Meagre (*Argyrosomus regius*). The aim of this was to test behavioural responses to stress, and attempt to determine whether differences in dietary lipid inclusions result in different behavioural responses and recovery rates to a standardised stress event, more specifically, hypoxia.

During the month of January, Caroline Navjord (master student on Aquaculture in Nord University) resumed her experiment on the effects of size on optimal swimming speed and cost of transport in rainbow trout (*Oncorhynchus mykiss*), for a Short Term Scientific Mission funded by a grant from COST Office (Food and Agriculture COST Action FA1304: Swimming of fish and implications for migration and aquaculture; FITFISH). However, due to a number of complications, Caroline could no longer stay in Hirtshals to finish the project, having it being taken over by myself in the end of the month. This project lasted for the remainder 3 months and is further described later on the report. The study focused on trying to understand what are the effects of size on optimal swimming speed (Uopt) and cost of transport (COT) in this particular species of trout, and the implications for their use as variables in exercise induced growth improvement.

Effect of dietary fatty acids and phospholipids on fast-escape response and rheotaxis of juvenile Pikeperch (*Sander lucioperca*)

Introduction

Pikeperch (*Sander lucioperca)* is a strong candidate for the potential diversification of intensive freshwater recirculation aquaculture systems (RAS) farming in Europe (Dalsgaard et al., 2013). However, the relatively limited knowledge regarding Pikeperch fatty acid requirements during the larval phase and their high susceptibility to stressors are major holdups for its further expansion (Lund et al., 2014). In modern intensive aquaculture, the robustness and stress resilience of the fish are of crucial importance in terms of welfare, health, growth, quality of the end product and thus overall production costs (Lund et al., 2014).

A prerequisite for most successful fish larval rearing is an optimal fatty acid composition of the diet. Adequate supply of HUFAs during early developmental stages of larval fish is essential for the normal progression of neurogenesis (Mourente, 2003), and dietary supplementation with phospholipids increases the health status of farmed Pikeperch (Hamza et al., 2008a; Henrotte et al., 2010; P. Kestemont et al., 1996; Lund et al., 2012). A recent study on feeding Pikeperch larvae with monounsaturated fatty acids from olive oil caused dysfunctions such as increased stress sensitivity and mortality as well as reduced salinity tolerance (Lund & Steenfeldt, 2011), which indicated the importance of long chain polyunsaturated fatty acids (HUFAs) provided in the diet. Moreover, diets deficient in HUFA, particularly DHA, during first feeding are associated with a suite of negative consequences, one of which is brain developmental disorders (Lund et al., 2012; Lund & Steenfeldt, 2011), suggesting that DHA is crucial in cognitive function and stress responses in Pikeperch (Lund et al., 2014).

Presently, dietary HUFA intake in fish is loosely linked to neurophysiological function and behavioural stress responses, due to the limited amount of evidence and information on the subject (Benítez-Santana et al., 2012). Recent studies on Gilthead seabream larvae (*Sparus aurata*) provided some direct evidence and effects of n-3 HUFAs on Mauthner neurons (Benítez-Santana et al., 2014; 2012). Larvae fed low levels of DHA displayed a tendency towards delayed escape responses (latency time increased) and significantly slower peak acceleration rates during escape responses following a mechano-sensory stimulus. Pikeperch are presumed to lack the ability to elongate fatty acids thus, we can

presume its dietary requirements may be similar to those of marine carnivorous fish species (Mourente, 2003; Salze et al., 2005), and therefore suffer from similar drawbacks.

To further improve our knowledge concerning Pikeperch and its dietary requirements, the present work aimed to investigate the effects of increasing dietary inclusion of phospholipids and additional effect of n3-/n6- HUFA ratios on Pikeperch larval and/or juvenile physiology and welfare indicators, such as ontogenetic development and performance. During this task, the neural development was measured using visual and mechano-sensory acuity during avoidance responses and rheotactic behaviour. Therefore, we focused on the hypothesis that dietary fatty acid compositions and phospholipid content in larval feed can both affect fast-escape response to mechano-sensory stimuli and rheotaxis in the fry stage. The fast-escape response can be divided into several distinct components. Overall, it's a sensory component in which the fish senses a threat by means of the visual, acoustic or mechanical sensory system (Marras et al., 2011). The sensory output of these systems is transmitted to a pair of reticulospinal neurons, the Mauthner cells, particularly related to escaping behaviour in fish (Benítez-Santana et al., 2012), which in turn elicit a behavioural response (Eaton et al., 2001). The fast-escape response can be analysed for a variety of variables such as response latency, escape trajectory, velocity, acceleration and more (Domenici et al., 2007). Failure to execute an escape response in a timely fashion may be caused by either failure to properly sense a potential threat (sensory failure) or failure of the Mauthner cells to properly trigger a behavioural response (response failure) (Lund et al., 2014). Proper function of both these elements has been shown to be influenced by dietary supply of HUFAs (Fuiman & Ojanguren, 2011).

Rheotaxis, the ability to orient upstream to current, is the other way mechano-sensory stimuli will be analyzed in this study. Rheotaxis relies on the neuromasts of the lateral line system (Montgomery et al., 1997), and the basis of this sensory system is an array of receptor units called neuromasts, which contain clusters of hair cells along with support cells. Upon deflection, hair cells increase transmitter release and excite afferent neurons (Dijkgraaf, 1963). Behaviours such as schooling (Partridge & Pitcher, 1980), prey detection (Hoekstra & Janssen, 1985; Montgomery & Hamilton, 1997) and escape (Blaxter & Fuiman, 1989; Feitl et al., 2010) have been shown to be dependent on the proper function of the lateral line system. Although rheotaxis in adult fishes has received much attention (Harris et al., 2003; Hernández et al., 2006; López-Schier & Hudspeth, 2006; Williams & Holder, 2000), very little is known about rheotaxis and related behaviours in larvae and juveniles.

Materials and methods

Diets

Six different diets with increasing content of phospholipids, DHA and EPA levels were tested, as shown in Table 1. Cold extruded feed was prepared as 0.4 mm and 0.8 mm by SPAROS, Portugal.

Table 1: Dietary composition and analytical content of the 6 experimental diets.

Diet Ingredients (%)	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
MicroNorse Fish Meal	45	45	45	45	45	45
CPSP 90	7	7	7	7	7	7
Squid meal	13	13	13	13	13	13
Fish gelatine	1	1	1	1	1	1
Wheat Gluten	4.4	4.4	4.4	4.4	4.4	4.4
Wheat meal	6.1	5.9	5.6	6.1	5.9	5.6
Algatrium DHA70	0.0	0.0	0.0	0.55	2.0	3.4
Olive oil	18.9	12.1	3.4	18.35	10.1	0.0
Vitamin & Mineral Premix PV01	1.0	1.0	1.0	1.0	1.0	1.0
Soy lecithin powder	3.0	10.0	19.0	3.0	10.0	19.0
Binder (guar gum)	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant powder (Paramega)	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant liquid (Naturox)	0.2	0.2	0.2	0.2	0.2	0.2
Analysed content (% DM)						
% dry matter (DM)						
Crude protein	54.1	54.7	55.6	54.1	55.8	55.3
Crude lipid	26.8	25.9	24.6	27.6	25.6	24.8
NFE + fibre (substracted)	3.0	3.0	2.8	2.8	3.1	3.2
Ash	9.1	9.4	10.0	9.0	9.3	10.2
PC (phosphatidylcholine)	1.40	2.61	4.31	1.42	2.68	4.29
PE (phosphatidylethanolamine)	0.43	1.22	2.20	0.40	1.14	1.87
PI (phosphatidylinositol)	0.44	1.28	2.44	0.43	1.28	2.48
TPL (Total phospholipids)	3.73	8.19	14.38	3.70	8.32	14.51
DHA (% TFA)	3.6	4.2	5.1	5.2	11.7	21.4

Larval rearing and feeding scheme

Eggs were obtained from AquaPri April 2016 and were hatched in an incubation system (two 0.5 m^3 tanks) at DTU Aqua at ambient temperature of approximately 18^oC and gradually increased to 19-20^oC. From dph 3-15 larvae were fed with newly hatched unenriched *Artemia nauplii*, kept alive in a suspension and fed continuously for 10-12 hours by a feed dispenser. At 15 dph -20 dph larvae were fed a mix of the 6 above dry feeds and artemia were slowly removed by postponing the feeding of these. At dph 20 they were moved to a temperature controlled flow through larval system consisting of 16 square tanks of 300L each. The experiment was carried out in a triplicate set up with 3

tanks per feed type (6 x3) (Figure 4). Each tank was initially stocked with approximately 500 larvae and kept at 19-20 °C, in fresh water.

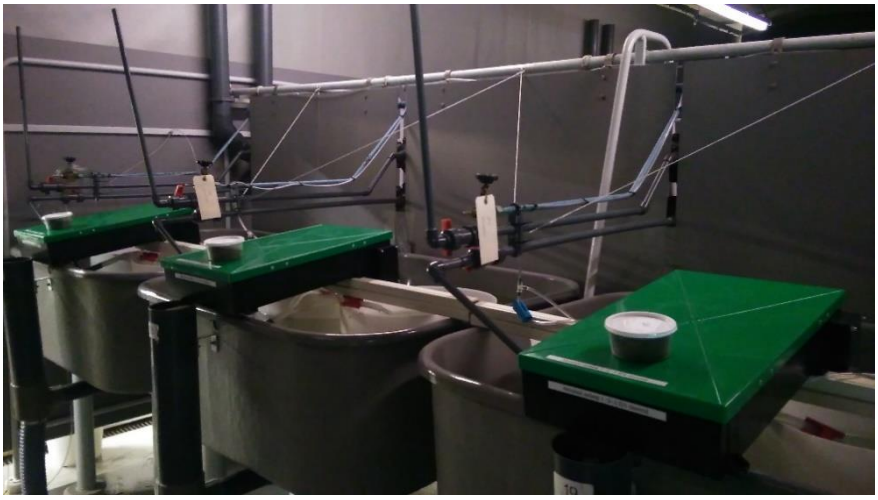

Figure 4: Juvenile Pike perch tanks, North Sea Science Park, Hirsthals.

From 20 – 40 dph, juveniles in each triplicate groups were fed one of the six experimental diets. The manufactured feedtypes were 400, 600, and 800 µm. During the first week 400-600 µm (grinded to 200-400 µm) was used, gradually exchanged with 800 µm during the second week and used for the remaining of the experiment. Feed was given by automatic feeders (daily from 8-20h). Larvae were fed dry feed in surplus approximately 25% (of expected larval biomass) in the first week decreasing to 10 % during the fourth week. Tanks were vacuum cleaned daily for dead larvae, faeces and remaining feed.

Fast-response

The experiments were conducted on juveniles in a white semi-translucent polyethylene circular tank, covered in black opaque plastic to prevent visual disturbance of the fish, as shown in Figure 5. A black cylindrical piece in the middle of the tank was also included in order to attract the fish to that area and therefore making it easier to get good recordings of the fast-response. Fish were transferred to the tank without air exposure and allowed to acclimatize to the tank for a period of 40 minutes. Video recordings were made at a rate of 240 fps using a Casio high-speed camera mounted 80cm above the water surface. The setup was illuminated from below using a LED light panel. The escape response was triggered by mechanical stimulation by releasing an iron nut manually from a height of 85-90cm above the water surface, inside a vertical PVC pipe suspended approximately 1cm above the water surface. To avoid differences in performances due to different distance from the stimulus, the fish were only stimulated when within range of the pipe and away from the walls of the tank. Fast-escape was

determined three times for each juvenile, with 20-40 minutes to let the fish recover between tests. There was no water replacement or current during experiments. Escape responses were analysed using Tracker (v. 4.95, http://physlets.org/tracker/). Response latency (time between the nut hitting the water surface and the escape response), escape speed and acceleration (within the first 20 frames since the start of the escape response) were the assessed parameters.

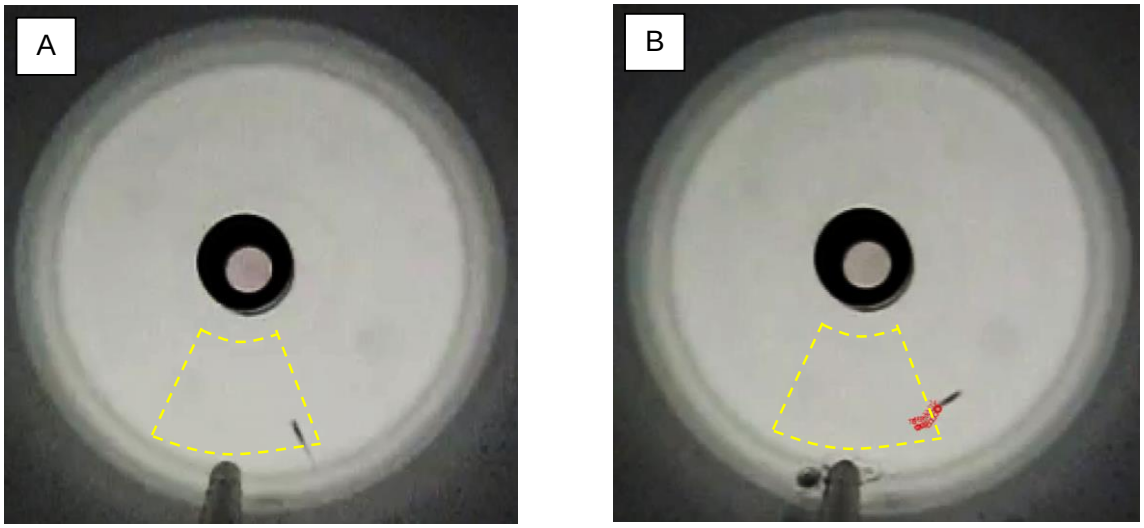

Figure 5: Fast-response experiment set up. Pike perch were put in a white semi-translucent polyethylene circular tank, covered in black opaque plastic, filled with some water. In the centre is a black cylindrical piece in the middle of the tank to attract the fish towards the area inside the yellow lines (as shown in A), and on the bottom is the PVC pipe from which the iron nut was dropped. Escape responses were analyzed using Tracker (v. 4.95, http://physlets.org/tracker/), as seen by the red dots in (B).

Rheotaxis

This trial was conducted on Pikeperch juvenile in a white opaque plastic rectangular flume channel measuring 5 × 5 × 50 cm, as shown in Figure 6. The water was pumped directly from the tank where the fish resided. It came in from one side through 10 individual tubes of identical size, which were opened and closed to change the flow speed inside the flume, and later returning to the same tank. The flume was fitted with 2 honeycomb baffles, one in the front to keep the flow steady and even, and another in the back, to prevent the fish from washing out. Water flow was calculated using a 500ml beaker and a stopwatch, counting how many seconds it took to fill up with water while one tube was open, and repeating the procedure every time an additional tube was open (all tubes were numbered and opened in the same order). The fish were transferred to the flume without air exposure and left without current for about 30min to acclimatize. The tubes were opened in succession to steadily increase flow (approx. 10ml/s per tube) while the fish's rheotactic response was waited for. For the purpose of this experiment,

rheotaxis was defined as the moment when the fish aligned itself with the current for at least 5 seconds. At this moment, the number of tubes opened was registered, giving us the approximate flow at which rheotaxis occurred. After 30min, one tube was opened, followed by another every 5min, until rheotactic behaviour was seen, ending the experiment 10min after. The experiments were filmed using a Go-Pro Hero 3 set directly above the flume.

Figure 6: Rheotaxis experiment set up. On the right are the tubes where the water flows in, and on the left is a hole where it falls back in the Pike perch tanks. This way we ensured the water temperature remained the same throughout the experiment.

Unfortunately, the results from these experiments were not included in this report, and were instead used by Ivar Lund and the team for further analysis.

Hypoxia trial on Meagre (*Argyrosomus regius*)

Introduction

Meagre (*Argyrosomus regius*) belongs to the *Sciaenidae* family and it is a carnivorous species that inhabits the Mediterranean and the Black Seas, and also occurs along the Atlantic coast of Europe (Whitehead et al., 1986). The fact it can adapt very easily to captivity, exhibiting a high growth rate and good feed conversion ratio (Jiménez et al., 2005), and has the ability to tolerate wide ranges of temperature and salinity, makes it a suitable candidate species for the diversification of aquaculture in the Mediterranean region (Quéméner et al., 2002). Moreover, its lean and high lipid quality flesh makes meagre a species with high nutritional value (Grigorakis et al., 2011; Monfort, 2010; Piccolo et al., 2008; Poli et al., 2003; Quéméner et al., 2002). However, there is little information available on the dietary requirements of meagre, particularly on dietary lipid requirements.

The aim of this study was to test behavioural responses to stress, and attempt to determine whether differences in dietary lipid inclusions result in different behavioural responses and recovery rates to a standardised stress event, more specifically, hypoxia.

Methods

All fish, weighing between 150-250g, were subjected to a standardised stress protocol consisting of a 3-minute low water challenge where fish were subjected to a drop in water level in the holding facility so that they were forced to lie on their side, and subsequently transferred to a net, in which they were suspended in air for 3 minutes. During these 6 minutes, the behaviour of each fish was recorded on video. Following this, fish were returned to an aquarium and their swimming behaviour was monitored until they had resumed normal position in the water column. The objective was to try to determine whether fatty acid nutritional history had an effect on the stress response and subsequent recovery in individual fish. Variables used to correlate dietary influence was struggle type and duration during stress, time required to regain equilibrium, and time required to return to normal swimming. The trial tested behavioural responses to stress, and attempted to determine whether differences in dietary lipid inclusions resulted in different behavioural responses and recovery rates to a standardised stress event. We were purposely not informed about the composition of the three diets (C, D and F) throughout the full trial. Therefore we do not know how lipid content differed or what kind of lipid inclusions were being tested. Nevertheless, due to the close relationship between lipid

content and responsiveness to stress in fish, it seemed like an interesting and promising study.

Results and discussion

The trial presented no statistical differences across the 3 diets in any of the variables analysed, as shown in figures 7, 8 and 9. The way dietary lipid inclusion affects stress in meagre is still not very well understood, but a number of studies have been conducted to better understand how they can affect other marine species. Low dietary ratios of n-6 to n-3 fatty acids (Sargent et al., 2002) and low ratios of palmitoleic acid (18:1n-9) to n-3 fatty acids (Izquierdo et al., 1989) have been hypothesized to play a role in increasing the stress resistance of marine fish larvae.

Results of a number of experiments identified DHA as the n-3 HUFA that conferred the greatest stress resistance in marine fish larvae, since high levels of EPA or other n-3 HUFA were not effective when DHA was limiting (Dhert et al., 1993; Izquierdo et al., 1989; Watanabe et al., 1989).

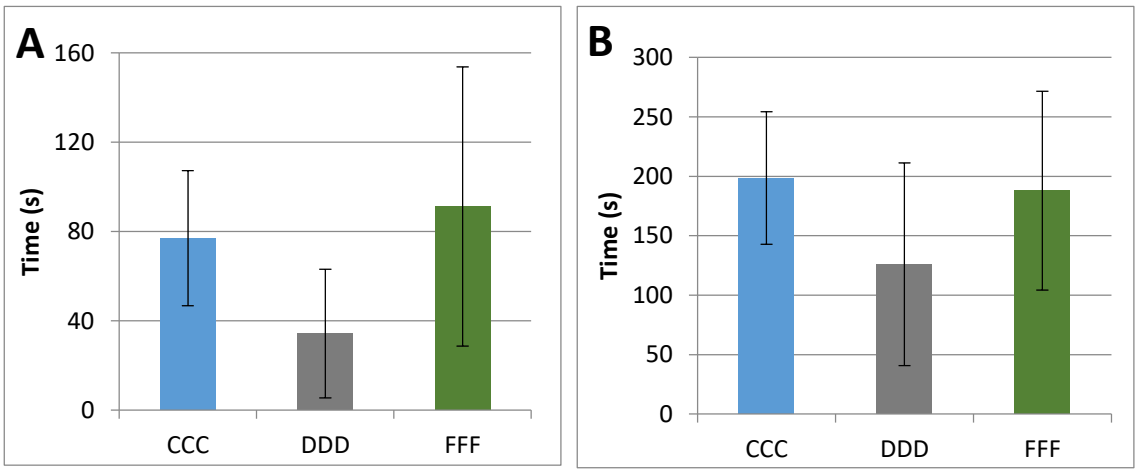

Figure 7: Results across 3 diets, (A) is Equilibrium recovery and (B) is Swimming control. The graphs represent the average and standard deviation of how long it took fish from each diet to regain equilibrium (A) and swimming control (B).

Also, variations in dietary fatty acid profiles because of the inclusion of vegetable oils may alter fish metabolism, which may affect fish stress resistance (Mourente et al., 2005). Studies with gilthead sea bream (*Sparus aurata*) showed an improvement in larval stress resistance to handling when dietary EPA was increased, which could be related to its possible role as a regulator of cortisol production (Liu et al., 2002). The same study showed that a reduction of EPA dietary levels reduced larval resistance to stress despite the presence of high dietary DHA. Liu et al. (2002) concluded that an increase in dietary

EPA and DHA could increase stress resistance of juvenile gilthead sea bream by inhibition of the plasma cortisol levels induced by chronic, acute and repetitive stress.

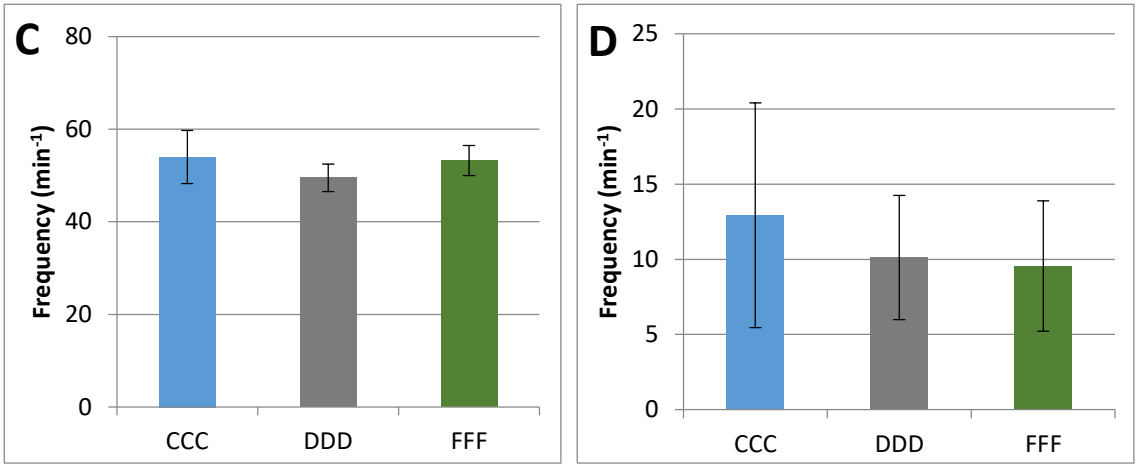

Figure 8: Results across 3 diets, (C) is ventilation frequency in low water and (D) is ventilation frequency in air exposure. The graphs represent the average and standard deviation of the ventilation frequency per minute while in low water (C) and exposed to air (D).

Furthermore, arachidonic acid (ARA) has been reported to be effective in reducing handling stress in gilthead sea bream larvae (Koven et al., 2001), but they appeared to be more susceptible to a chronic stress induced by daily salinity fluctuation, compared to fish fed lower dietary ARA. Previously, Kanazawa (1997) showed dietary DHA, soybean lecithin and phospholipids were effective in increasing the tolerance of red sea bream and marbled sole to the various stress conditions.

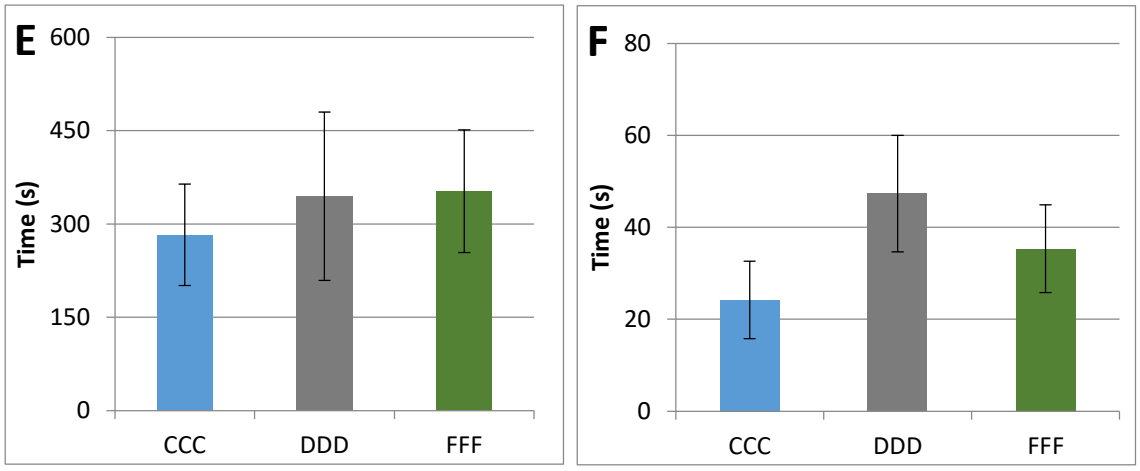

Figure 9: Results across 3 diets, (E) is buoyancy recovery and (F) is time spent struggling. The graphs represent the average and standard deviation of the time it took fish to recover buoyancy and the sum of time spent struggling.

This study analysed the duration and characteristics of the stress response, and it may be that those variables don't show the full picture and there are better ways to study this responses (for example, through cortisol measures). Despite the extensive body of

literature describing the impact of dietary fatty acids on fish response to stress in either larvae or juveniles, our work did not find significant differences between dietary treatments. The lack of differences suggests fatty acids have little or no impact on the responsiveness of fish to stress, but it could just mean that (1) the duration, intensity and recovery of the stress response did not show the true effects of lipid inclusions on stress, or (2) the type of lipids used were not the most impactful on the stress response.

Effects of size on optimal swimming speed and cost of transport in rainbow trout (*Oncorhynchus mykiss*), and implications for their use as variables in exercise induced growth improvement

Introduction

As aquaculture quickly grows, its focus on using aerobic endurance training to improve growth and reduce stress in fish farming is increasing. For many fish species, including salmonids, the calculated optimal swimming speed (Uopt) is thought to coincide with the ideal swimming speed for growth (Davison & Herbert, 2013). The Uopt for rainbow trout with a mass of ~250g is generally assumed to be 1.0 – 1.1 body length/second ($BL.s^{-1}$), and is usually considered to be the most efficient in terms of energy use (Walker & Emerson, 1978; Webb, 1971; Weihs, 1973). Still, there seems to be a general misunderstanding about Uopt in relation to fish size, and we aim to clarify those implications.

Uopt is calculated based on the metabolic cost (oxygen consumed) to travel a particular distance, and can therefore be affected by many factors, such as temperature, endothermy, other aspects influencing standard metabolic rate, and fish size (Beamish, 1979; Tudorache et al., 2007; Webb, 2005; Weihs, 1973). Therefore, research on optimal swimming speeds can be uncertain and equivocal when some of these factors are not accounted for.

The work conducted seeks to add to our understanding of the concept of optimal swimming speed and cost of transport in rainbow trout, specifically relating to the size of the fish. The aim is to investigate swimming performance in rainbow trout over a 7-fold size range, from 50 grams to 350 grams in body mass, corresponding to approximately a 1.7-fold difference in length.

While Brett (1964) determined the relationship between size and oxygen consumption during sustained swimming speeds in sockeye salmon, no effort has been made to determine how fish size (length) influences Uopt and cost of transport (COT) in rainbow trout. In general, greater sizes are associated with a reduction in relative critical swimming speed (Ucrit) (Brett, 1964; Hawkins & Quinn, 1996) and an increase in fatigue

time in fixed-velocity trials (Beamish, 1979; Brett, 1967; McDonald et al., 1998). We hypothesize that rainbow trout of smaller size are able to achieve higher maximum swimming speeds, and have correspondingly higher optimal swimming speeds, based on previous trials with zebrafish (Bagatto et al., 2001), dace, and trout (Bainbridge, 1958). Assuming that this hypothesis is true, a secondary objective of the study was to determine what the determining factors are for a higher swimming capacity in smaller fish (more efficient swimming by greater stride length), whether due to an ability to achieve higher tail beat frequencies, larger tail beat amplitudes, and/or greater relative investment in red muscle mass. These speeds should be, at least in part, achieved by the ability of smaller fish to sustain higher tail beat frequencies, and possibly by marginal changes in stride length (Bainbridge, 1958). Lastly, we'll try to perceive whether smaller fish have a higher relative investment in red muscle fibres than larger fish do, since the consensus is that exercise at higher speeds is powered mainly by aerobic red muscle (Hudson, 1973; Moves & West, 1995; Webb, 1971; Wilson & Egginton, 1994).

Materials and Methods

Animal Husbandry

Fish were obtained from a commercial fish farm (Funderholme Dambrug) and quarantined for 2 weeks in 15ppt sea water. Following quarantine, fish were randomly distributed in groups of 100 individuals among twelve circular polyethylene tanks with a water volume around 600L, identical to those previously described by Larsen et al. (2012). The tanks were connected to a common water supply within a recirculation bio-filtered system that delivered a 50 L min^{-1} flow of aerated freshwater at a constant temperature of 15°C to each tank by means of a centrifugal pump (Grundfos TP 25-90/2, Grundfos DK A/S, Bjerringbro, Denmark) fitted to each tank. Water quality parameters (NO^-_3, NO^-_2, NH_3/NH^+_4, pH) were monitored daily and did not exceed safe levels throughout the course of the study (NO^-_3 <100mg l^{-1}, NO^-_2 0–1mg l^{-1}, total ammonia NH_3/NH^+_4 0–1mg l^{-1}).

Photoperiod was maintained at 14 h: 10h light: dark (lights on at 7:00) throughout the experiment. Feeding was delivered by automatic belt feeders for periods lasting 8h (between 8:00 and 16:00). The feeding regime was set up from G. Rasmussen and From (1991) growth model based upon energy flow and partitioning parameters estimated from tank experiments with rainbow trout. This model defines feeding level *f* as the fraction eaten of the maximum quantity which could be eaten ($0 \leq f \leq 1$). The feeding level chosen for the growth trial was 0.84, corresponding to an initial daily ration of 1.3% of the estimated tank biomass and gradually reduced to a more restricted regime of 0.9% of

the biomass. The feed consisted of 3mm and later 4.5mm extruded pellets (42–47% protein, 28–32% fat, 12–13% carbohydrate; EFICO Enviro 920, Biomar A/S, Brande, Denmark). Faeces and debris were removed through a central drain in the bottom of the tank connected to a swirl separator. All use of animals for these experiments was in accordance with Danish and EU legislation.

Swimming experiments

All specimens were food deprived for 48 h prior to experimentation to remove any confounding effects on metabolic rate (Ross et al., 1992; Thuy et al., 2010) and were maintained at 15 °C during the entire respirometry period. Swimming performance tests were carried out in a 31.45 litre Brett type swimming respirometer designed to exercise fish in a non-turbulent water flow as described by Steffensen et al. (1984). A total of 33 fish were used ranging in mass between 54.2 and 375.5 grams. Fish were weighed and measured, and then transferred to the swimming respirometer. Water flow in the respirometer was controlled by a variable speed DC motor. Water flow ($cm.s^{-1}$) in the swimming section of the respirometer was calibrated using a vane wheel flow sensor (Höntzsch GmbH, Waiblingen, Germany) against the voltage output from the motor, which was collected by a PC running respirometry software. Swimming speeds were calculated as body lengths per second ($BL.s^{-1}$) and corrected for blocking effect as described by Bell and Terhune (1970) upon completion of the experiments. Oxygen consumption was measured in 10 min cycles consisting of a 4-minute flush period, 1-minute wait and a 5-minute measuring period. Individuals were inserted in the respirometer during the afternoon and allowed to acclimatise until the following morning at a water velocity of 6 cm s−1 (~0.5 $BL.s^{-1}$). The following day the swimming velocity was gradually increased in 0.5 $BL.s^{-1}$ increments with 3 oxygen consumption measurements at each velocity until fatigue (once the fish laid against the back grid for at least of 2 seconds). During all swimming trials fish were shielded from visual disturbance by a black plastic curtain. A small slit in the plastic towards the caudal end of the fish allowed for visual observation of the fish. Critical swimming speed was calculated according to Brett (1964), as $U_{crit}=U_{max}+(t/\Delta t)\times\Delta U$, where Umax is the speed of the fastest swimming trial which the fish could complete, t is the time the fish could swim at the swimming speed leading to fatigue, Δt is the duration of one swimming trial, and ΔU is the swimming speed increment. The energetics of swimming was calculated using cost of transport (COT). In order to integrate energy requirements of fish, COT was also calculated as kJ kg−1 km−1 using a conversion factor of 14.06 (Dejours, 1981). A polynomial equation (ax^2+bx+c) was fitted to the relationship between fish swimming speed and oxygen consumption. The swimming speed with the lowest

cost of transport and the corresponding oxygen consumption was calculated from the roots of the derivative as x=−b/2a and y=−(b2−4ac)/4a, respectively. Only swim trials where all three measurements had been completed were included in the data set. Maximum metabolic rate was considered to be the highest oxygen consumption measurement recorded during a measurement cycle where the fish swam continuously. O_2 saturation in the respirometer was measured constantly by a Pre-Sens oxygen meter (Presens, Germany) connected to a custom made dipping probe. O_2 saturation was measured constantly across three cycled phases (flushing, wait and measure). Video recordings were made for every fish at each speed for a minimum of 1 minute, to later analyse tail beat frequency and amplitude. At the end of each experiment, fish were sacrificed and transversely sectioned at the level of the pectoral fins, centre of the dorsal fin, and the anal fin. Section surfaces were photographed and used to calculate rations of white and red muscle fibres. The system was not shrouded and the fish were exposed to a normal light regime for the entire duration of the experiment.

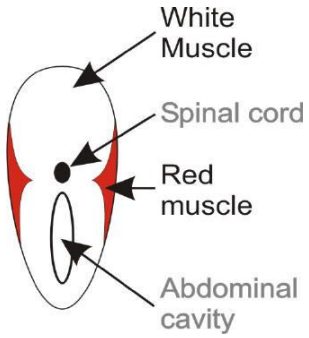

Figure 10: Schematic illustration of the transverse section of a teleost fish, showing distribution of red and white muscle fibres.

The analysed variables were body mass (g) (BM), standard length (cm) (SL), hepatosomatic index (HSI), viscerosomatic index (VSI), tail beat frequency (Hz) (TBF), tail beat amplitude (size index) (TBA), blocking index (BLOCKING), stride length (BL) (STRIDE LENGTH), and red muscle index in the front (RM1), middle (RM2) and caudal section (RM3). Blocking index represents the blocking effect of the fish's body in the swim tunnel, and was calculated as the fraction of cross sectional area with standard length. The red muscle index was calculated as the amount of red muscle in total muscle area (red and white muscle), as shown in Figure 10.

Statistical analysis

All differences in variance between groups were evaluated using F-tests, and single linear regression analysis by using the "least squares" method was used to compare all variables with Uopt and Ucrit (BL/s). Further statistical analyses will be made using R for a future publication of the work performed.

Results

This experiment was undertaken to understand how different sizes influence swimming ability in rainbow trout. In order to evaluate the different effects of the variables in swimming, we made linear regressions of all previously mentioned parameters.

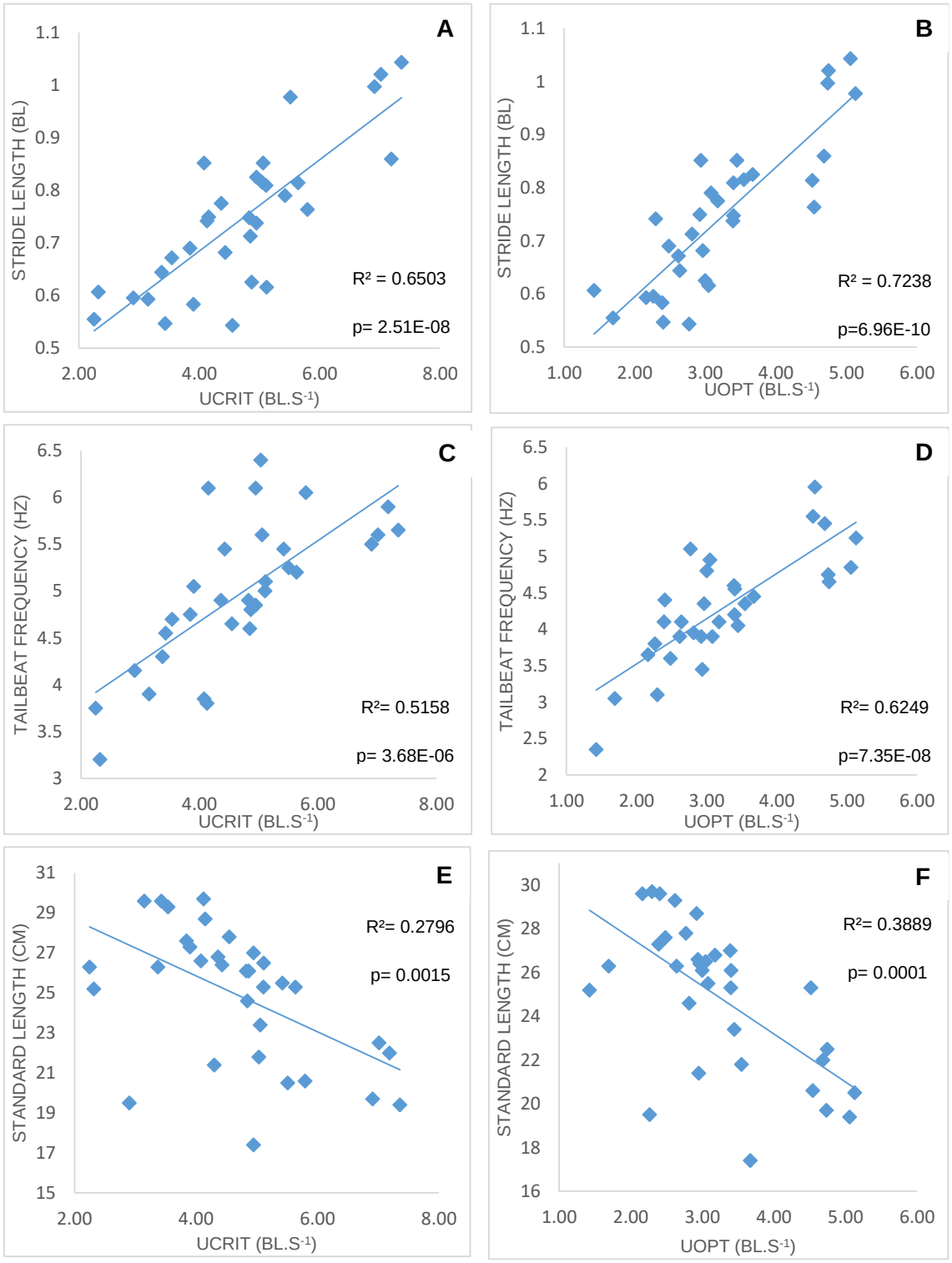

Figure 11: Relationships between (A and B) Ucrit/Uopt and Stride Length, (C and D) Ucrit/Uopt and TBF, (E and F) Ucrit/Uopt and Standard Length.

The most significant variables for both Uopt and Ucrit are shown on Figure 11, 12 and 13. Both Uopt and Ucrit showed similar interactions with the variables in question. Ucrit was found to be significantly negatively correlated with body mass, standard length, and blocking index, and positively correlated with stride length and tailbeat frequency ($p<0.05$). No correlation was found between Ucrit and HSI, VSI, TBA, and any of the three red muscle indexes ($p > 0.05$). Uopt had similar results to Ucrit, the only difference being the absolute values of each variable.

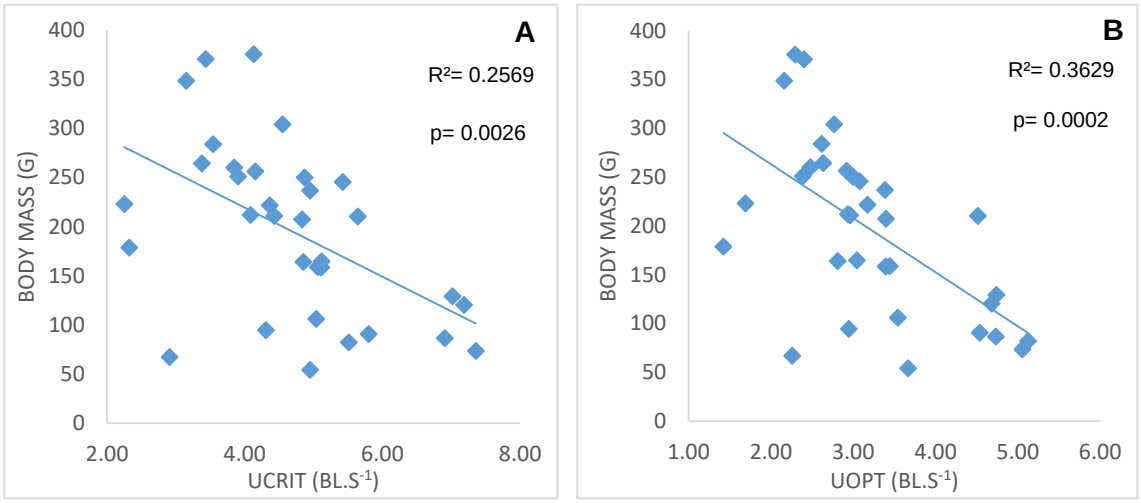

Figure 12: Relationship between Ucrit/Uopt and body mass.

A bigger stride length and higher TBF were found at greater Uopt and Ucrit. However, stride length seems to scale similarly between Uopt and Ucrit, which suggests this variable has the same investment at optimal and critical speeds.

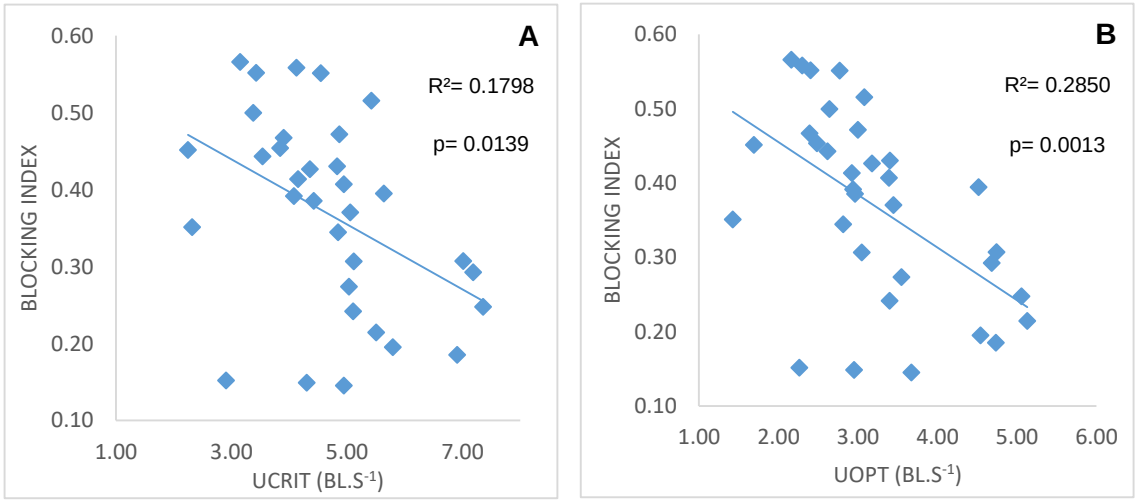

Figure 13: Relationship between Ucrit/Uopt with the blocking index.

Swimming speed is strongly related to SL ($R^2 = 0.2796$, $p= 0.0015$ for Ucrit; $R^2 = 0.3889$, $p= 0.0002$ for Uopt), and the same was seen for TBF ($R^2 = 0.5158$, $p= 3.68E-06$ for Ucrit; $R^2 = 0.6249$, $p=7.35E-08$ for Uopt), Stride Length ($R^2 = 0.6503$, $p= 2.51E-08$ for Ucrit; $R^2 = 0.7238$, $p=6.96E-10$ for Uopt), BM ($R^2= 0.2569$, $p= 0.0026$ for Ucrit; $R^2= 0.3629$, $p=$

0.0002 for Uopt) and Blocking (R^2= 0.1798, p= 0.0139 for Ucrit; R^2= 0.2850, p= 0.0013 for Uopt).

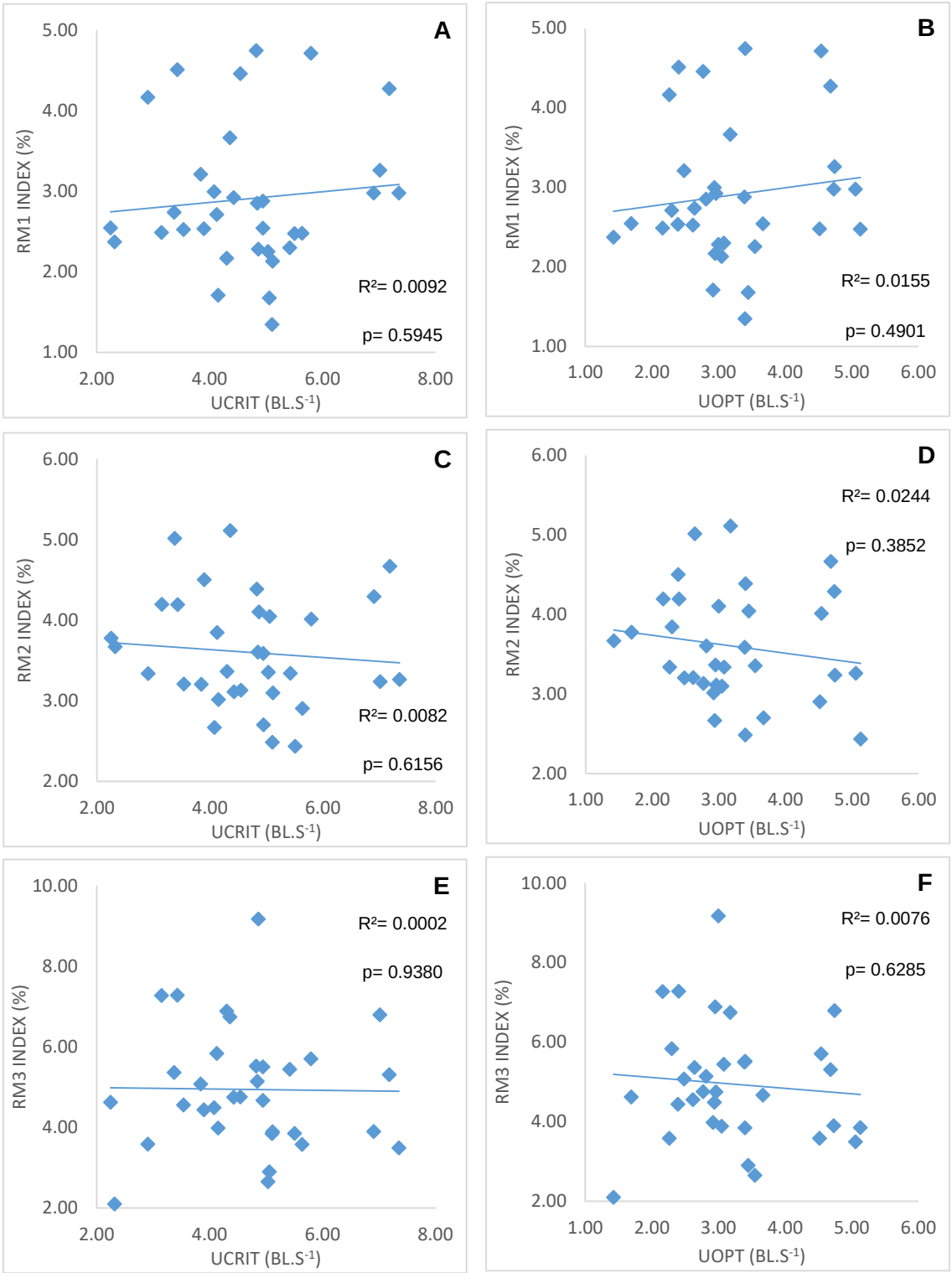

Figure 14: Relationships between (A and B) Ucrit/Uopt and RM1, (C and D) Ucrit/Uopt and RM2, (E and F) Ucrit/Uopt and RM3.

The amount of red muscle showed no correlation with swimming speed in any of the 3 different sections, as shown in Figure 14.

Discussion

This is one of the few studies aimed to evaluate the effect of size differences in swimming ability of rainbow trout. In a previous study on rainbow trout, Skov et al. (2015) found a significant negative effect of exercise at a current of 1 $BL.s^{-1}$ on both SGR and feed conversion ratio, and one of the possible explanations for it was because of the smaller size of their fish and the Uopt they chose to use, for trout with a mass of ~250g. They previously emphasized the need to consider what the Uopt is for the size of fish, because otherwise we won't ever be able to fully demonstrate and understand the physiological responses of rainbow trout to exercise.

In this study, our results showed that smaller fish have a noticeably higher Uopt and Ucrit, that ought to be accounted for in future experiments. However, the study's aim was not only to show how size affects swimming speed, but also which variables are more impactful.

Tailbeat frequency (TBF) seems to be the major drive for a faster swimming speed, and our results were in accordance to those obtained by Bainbridge (1958). TBF showed a strong correlation with size and speed, with smaller sizes achieving the highest values. Alongside TBF, stride length is also strongly correlated to swimming speed. The stride length of a fish is the distance covered per tail beat, and, according to Videler and Wardle (1991), it's a useful measure for comparisons between fish of different sizes and belonging to different species. At low speeds, fish have to use their pectorals and erect unpaired fins because it's harder to control steering, buoyancy and equilibrium, causing more drag and resulting in a smaller stride length (Videler & Wardle, 1991).

Drag always has an impact on swimming speeds, and its influence depends on the size of the fish. Velocity corrections are commonly used to compensate for the blocking effect of the fish on the flow in the tunnel (Videler & Wardle, 1991). The faster swimming speed of smaller fish should be partially explained by the reduced resistance their bodies offer. Our results show that higher Blocking indexes are associated with lower speeds and larger fish, and the opposite for smaller Blocking values.

Tail tip amplitude would also seem to be an important factor to account for. According to previous studies, relative amplitude of the tail tip (amplitude over body length) is usually found to be constant over a wide range of swimming speeds, its value commonly being around 0.1 BL (Videler & Wardle, 1991). Our results were somewhat similar, with amplitudes averaging around 0.15 BL. We observed that amplitude did not have a linear correlation with speed and was more variable at low speeds, similar to Webb's (1984) observations. Fish instead focus on increasing frequency rather than amplitude to

achieve faster speeds, because, unlike frequency, amplitude is limited by size. Therefore, we concluded that TBA didn't have a significant impact on swimming speeds.

Muscle fibres differ in their function and structure and are, therefore, recruited at different swimming velocities (Bone, 1978). Red muscle is usually active during sustained swimming (Magnoni et al., 2013), which could indicate that a larger amount meant the fish could endure exercise for longer. This study saw no significant correlation between red muscle index and swimming speeds. There is not much we can take from that, but it's likely the relative investment in red muscle did not differ between smaller and larger fish. This allows us to say RM is not responsible for differences in swimming speeds between sizes, letting us focus on other more relevant variables to justify these correlations.

In general, a reduction in Ucrit and Uopt is associated with greater size, and there's a decline in the ability to sustain higher speeds as size increases. In the words of Brett (1965), this occurs despite a relative increase in body musculature and a progressive increase in metabolic scope, as we verified with RM index. These assets do not seem to keep up with the increased hydrodynamic drag resulting from a larger size. In order to sustain higher speeds, larger fish require greater amounts of energy, which they simply can't keep up with their metabolic processes.

So far, the study showed there were number of impactful aspects on swimming ability, and gave us an understanding on how each variable affects the swimming speed individually. It clearly illustrated the importance of accounting for size differences when performing a swim study, and how swimming is a complex process with many variables to be accounted for. Therefore, rainbow trout might not be the odd one out when it comes to benefiting from exercise training, and it could just be that size differences unaccounted for justify the suboptimal SGR and overall poorer growth seen in previous studies.

In conclusion, smaller trout can achieve higher critical swimming speeds than larger trout, and have a proportionally higher optimal swimming speed. The main reason for this appears to be the ability to reach higher tail beat frequencies in combination with a greater stride length, which in turn results from less hydrodynamic drag.

The next step on our statistical analysis is to understand the importance of each variable, when all are taken into account. We hope further work using R software may show us how the variables truly influence swimming ability of different sized rainbow trout, giving us a full picture for the betterment of future research on this species.

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